#16 Dda 9/9/03

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

Applicant	:	Graff et al.		
Serial. No	:	10/002,631	Examiner:	To Be Assigned
Filed	:	October 31, 2001	Group Art Unit:	To Be Assigned
For	:	METHOD TO IDENTIFY SIGNAL SEQUENCES		

DECLARATION UNDER 37 C.F.R. §1.131

Professor Jonathon M. Graff residing at 3124 Milton Ave, Dallas, TX, 75205 and Matthew R. Muenster residing at 2014 Royal Oaks Drive, Irving, TX 75060 declare as follows:

1. We are the applicants of the above-identified patent application and coinventors of the subject matter described and claimed therein.

2. Prior to March 9, 2001, we completed the invention described and claimed in the subject application, in this country. The following set of facts demonstrate conception and reduction to practice of the subject matter of the present invention prior to March 9, 2001, the filing date of Tan et al. (US2002/012755A1).

3. Prior to March 9, 2001, Matthew R. Muenster prepared a vector comprising an ampicillin resistance (AmpR) gene with the native signal sequence deleted as a backbone vector for cloning the vector of the present invention. A copy of the relevant notebook data pages is attached as Exhibit A. The dates from Exhibit A are redacted.

4. Prior to March 9, 2001, Matthew R. Muenster prepared a vector comprising genes encoding known secreted proteins, e.g. leptin, directionally cloned upstream of the AmpR gene. Matthew R. Muenster shows that host cells transformed with this vector survived on

selection media. Expression of a cDNA fragment encoding a protein comprising a signal sequence confers survival of the host cell on selection media. A copy of the relevant notebook data pages is attached as Exhibit B. The dates from Exhibit B are redacted.

5. Prior to March 9, 2001, Matthew R. Muenster confirmed the sequence of the vector comprising the leptin gene cloned upstream of the AmpR gene. A copy of the relevant notebook data pages is attached as Exhibit C. The dates from Exhibit C are redacted.

6. Each of the dates redacted from Exhibits A-C is prior to March 9, 2001. Furthermore, each of the dates redacted from Exhibits A-C is prior to March 30, 2001.

7. We declare further that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Jonathon M. GRAFF

Date: JMG 7.1.03

Matthew R. MUENSTER

Date: _____

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_____

Jonathon M. GRAFF

Date: _____

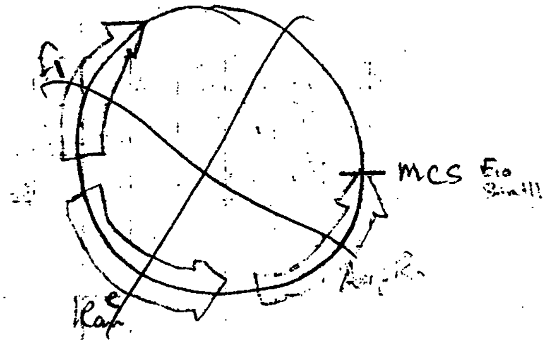
x Matthew R. Muenster

Matthew R. MUENSTER

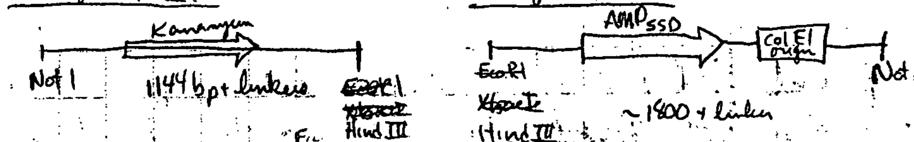
Date: x 7/9/03

Cloning of a bacterial ~~vector~~ vector to ~~generate~~ use for secreted protein screening.

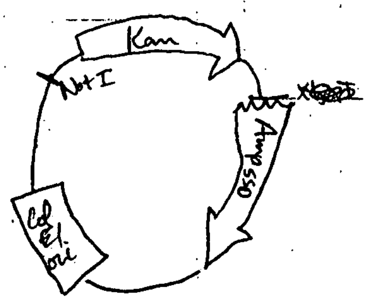
Strategy
1) PCR 2 distinct fragments from the original vector, which when ligated will give the desired vector



Fragment 1 will contain the kanamycin resistance gene ~~and the first part of the~~
Fragment 2 will contain the amp gene (w/o SS) and the Col E1 origin.



2) ~~Place~~ Ligate the two fragments to generate:

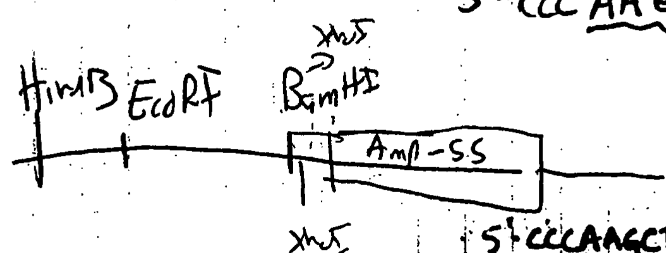


Now clone an dsgs from that introduces EcoRI, +XhoI sites ~~NheI, XbaI, SpeI~~ sites into the HindIII site. ~~Clone the NheI, XbaI~~

Kana 5' primer
5'-CCC ~~AG~~ AAGCT TCA GGGCGCAA GGGCTGC-3'

Kana 3' primer
5'-CCC AAGCTT ACTCTTCCTTTTTCAATTCAG

Size: 1162 bp



Amp 5' primer
5'-CCCAAGCTTGAATTC CACCCAGAAACGCTGGTG

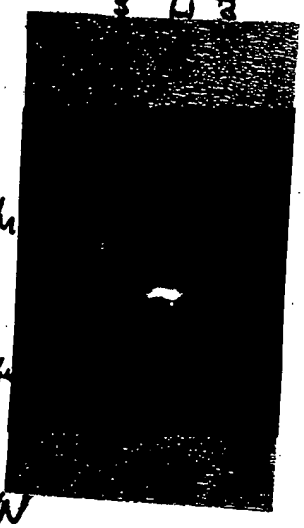
5'-Seq Primer
~~5'-CCTTCTATCGCCTTCTTG-3'~~
5'-CGCCGCTCCCGATTCGCA
GCGC

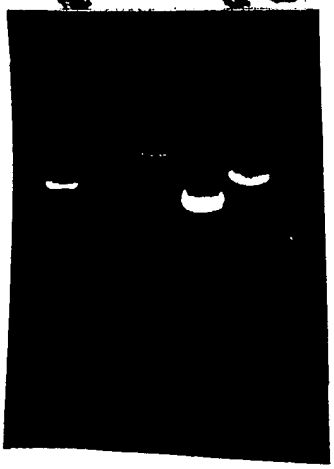
Amp 3' primer
5' CCC AAGCTT ATGTGAGCAAAA GGCCAGC-3'

Size - 1692 bp

some pCRII-TOPO.
had out of the
ning but they have.
added.
JTP's.
act 2
lenow
ater
otal 37°C 1hr

of this rxn.
2ul 5X Lig Buffer
' Ligase
ater.
tal 18°C ON





Either. BglII did not cut or there is no BglII site as the sequence says.

ed 5ul of this to 100 ul old " comp cells and plated the entire amount onto plates that JMP + I made. I got 0 / colony total and grew this up. ults are shown above.

Prepare to PCR fragments for use in cloning the bacterial secretion screen vector!

Set up the following Rxn's.

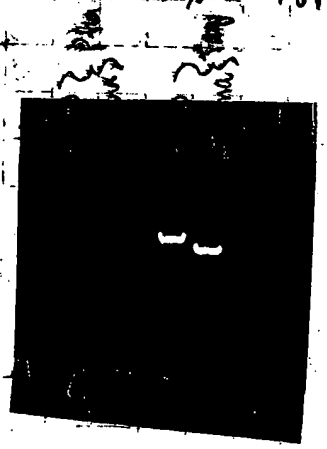
Pfu

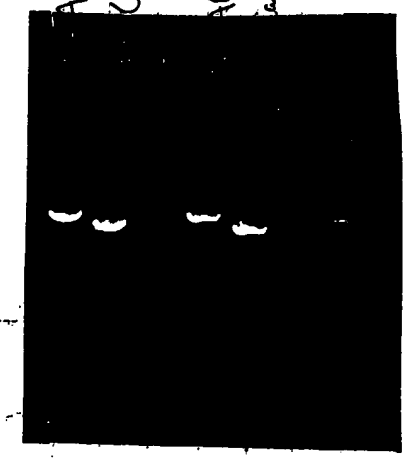
10 µl Pfu Buffer
4 µl dNTP's
1 µl template (100ng/µl
5 µl primer mix (50 ng/µl each)
1 µl Pfu Turbo
79 µl water
100 µl total.

Taq

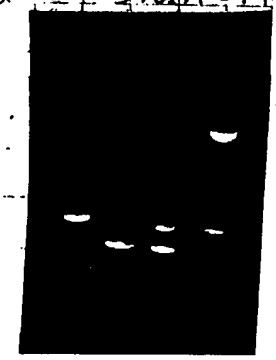
2.5 µl Taq Buffer
0.8 µl $MgCl_2$
0.2 µl template
1 µl primer mix
0.25 µl Taq
1 µl dNTP's.
19.25 µl water
25 µl Total

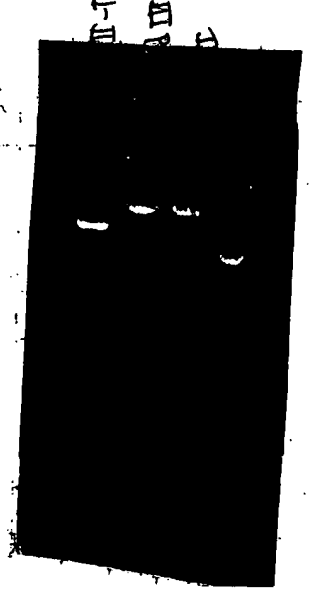
Rxn 1: - AO - Pfu.
2: Kan - Pfu.
3: + Cont - Pfu.
4: AO - Taq
5: Kan - Taq.
6: + Cont - Taq.





PCR cleaned the AO-Vent, Kan-Vent, and a Mix of the Taq Fragments. Eluted to 50 µl EB. I digested 25 µl of each eluate by adding 6 µl React II, 2 µl Hind III and 2 µl XbaI. 37° 1 hr. Also did test digestions to ensure that the enzymes worked. That is shown to the right. PCR cleaned the 3 rxn's. Ran 2 µl of each out on a gel.





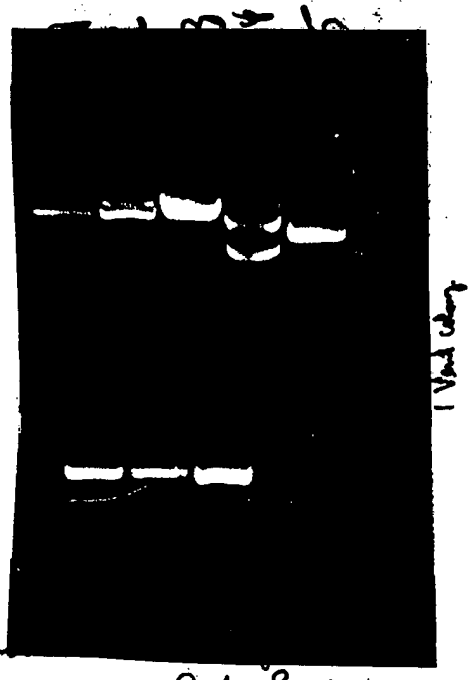
TA clone the Vent fragments in the following reactions.

8.

	AO-Vent-~~PCR~~A-tagged	Kana-Vent-~~PCR~~A-tagged	(P) Control	-Cont
Lig Buffer	5 ul	5 ul	5 ul	5 ul
DNA	1 ul	1 ul	2 ul (Control insert)	0 ul
M-T-Easy	1 ul	1 ul	1 ul	1 ul
DNA Ligase	1 ul	1 ul	1 ul	1 ul
H2O	2 ul	2 ul	1 ul	3 ul
	10 ul	10 ul	10 ul	10 ul

Plated all 10 ligations - pCRII & EB3 in ~~pCR II~~ pCR 2.1 to different plates.

	Amp	Kana	Kana+Amp	Hemk Kana
Vent -lig	—	0	0	—
Vent +lig	—	~~0~~ 1	0	—
Taq -lig	—	0	0	—
Taq +lig	—	0	0	—
SIDTP -lig	2	—	—	—
SIDTP +lig	~350	—	—	—
TA-AO	~300	—	—	—
TA-Kana	~500	—	—	—
TA +cont	~300	—	—	—
TA -cont	~40	—	—	—
pCRII xform	lawn of colonies	~200	~60	~35
EB3	lawn of colonies	~300	~250	~40
pCRII glycerol	—	lawn of col.	lawn of colonies	—



Picked 5 colonies from the TA-Kana-fragment plate and plated. Grew the AO's in LB+Amp and grew the Kana-clones in LB+Kana. Only 3 of the LB+Kana's grew. All five of the AO's grew. I minipreped these and digested them w/ Hind III. A1 & A2 appeared to be correct for the AO and all three clones of the Kana fragment K1, K2, K3 were correct. I cut out the bands, cleaned them, and set up ligations w/ them.

Did an EcoRI, BamHI digestion of pCRII to make a vector in which to clone my XcmI oligos so as to convert pCRII to a "homemade" TA cloning vector.

Either ① EcoRI did not cut or ② there is no EcoRI site in my clone.

Hypothesis 2 is consistent with the digest I did on 7/24 where I did an Eco/Bgl double digestion and concluded the Bgl did not work or its site was missing.

I need to sequence through the MCS of my clone to see what I got in the MCS.

apping the new construct w/ Rsa I.

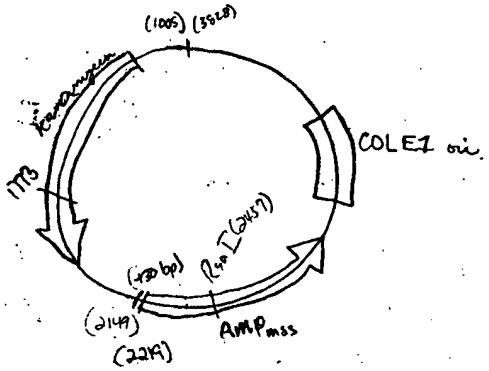
Rsa digests the parent plasmid @ 285 bps, 1773,6p, 2457 bp.
This yields fragments of 684, 1488 bp, & 1728 bp.

My new clone will include bases 1005-2149,
2219-3828.

There are 2 Rsa Sites in my new clone.

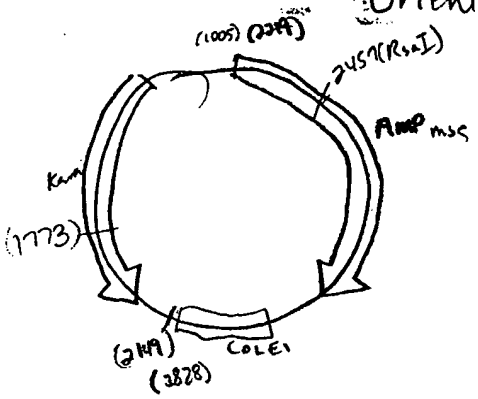
2 possible clones (orientations)

Orientation 1



Rsa dig yields 644 bp & 2140 bp.

Orientation 2.



Rsa yields 1030 bp & 1747 bp.

Setup the following ligations using the generated fragments:

	K1	K2	K3	A's	K's
2x Ligase Buffer	5	5	5	5	5
T4 Ligase	1	1	1	1	1
frag K1	1.0	0	0	0	0.33
frag K2	0	1.0	0	0	0.33
Frag K3	0	0	1.0	0	0.33
frag A1	0.25	0.25	0.25	0.5	0
frag A2	0.25	0.25	0.25	0.5	0
water	2.5	2.5	2.5	3	3
	10	10	10	10	10

Ligated @ RT for 1 hr.

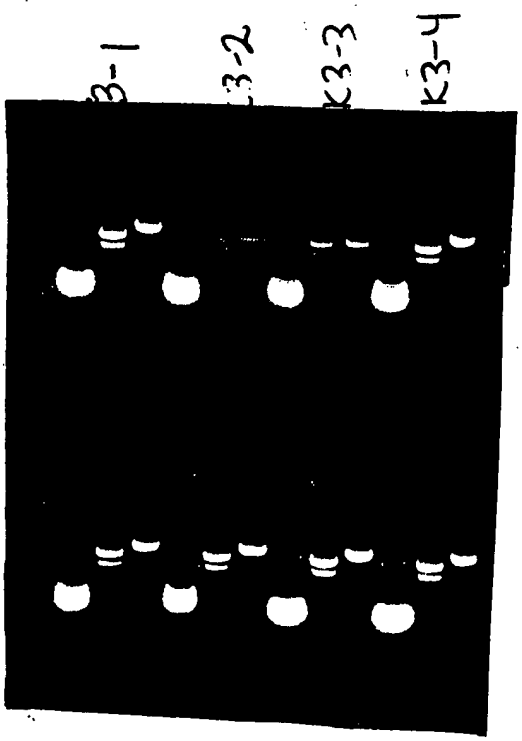
X-formed 20ul ONE SHOT's from Henk Lab w 5ul of each ligation
5 min, - 1 min · 2 min; 40 mins
Plated half of each tube to LB+ Kana; and LB+Kana+Amp plates.
Colony counts are shown below:

	LB+ Kana	LB+K+Amp
K1	13	0
K2	10	1
K3	32	0
A's	0	0
K's	0	0

Picked 12 colonies from the K3 plate and grew up in 5 mL LB + Kan.

Minipreped

Did HindIII and RsaI digests



K3-1,4,5,6,7,8 are correct in orientation 1.

K3-2,3 are correct and in orientation 2.

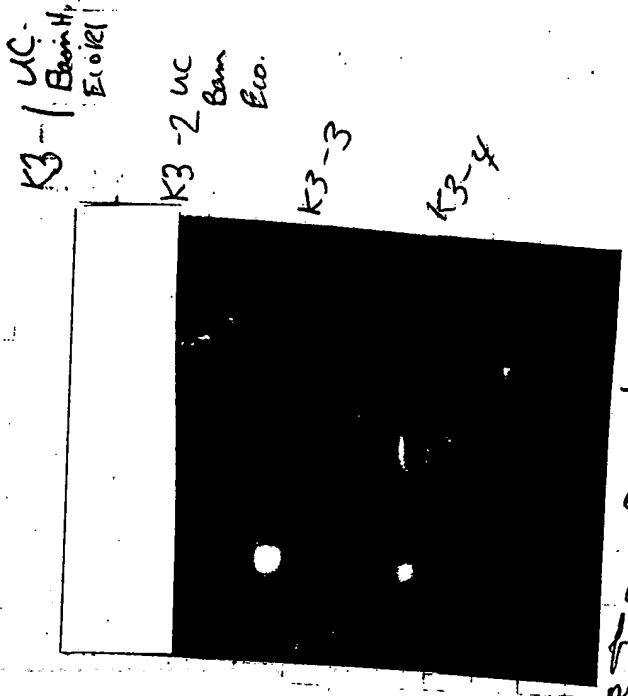
I will grow up K3,1-4. and midiprep them.

Student's Name

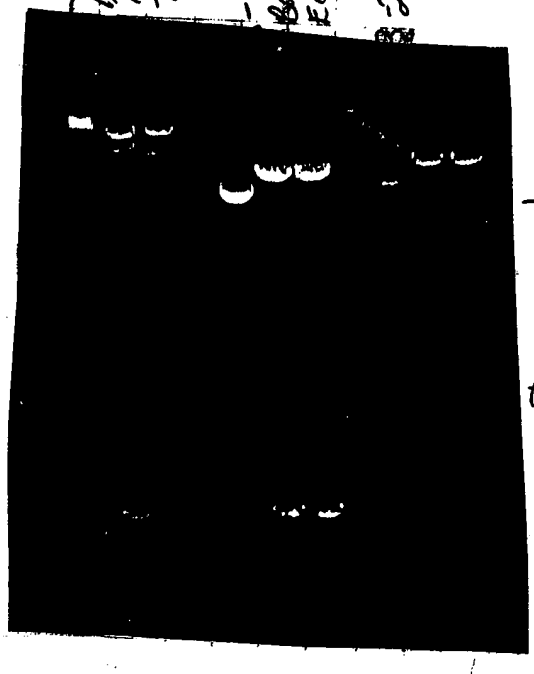
Subject

Dat

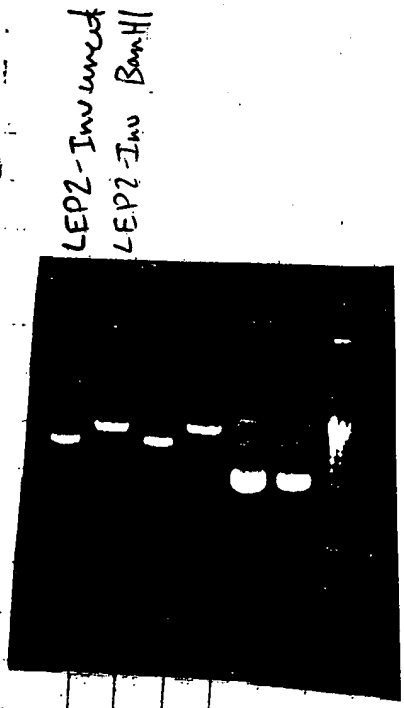
Instructor's Name



These look screwed up and different from the previous dig So I will do the midiprep and repeat the digestions

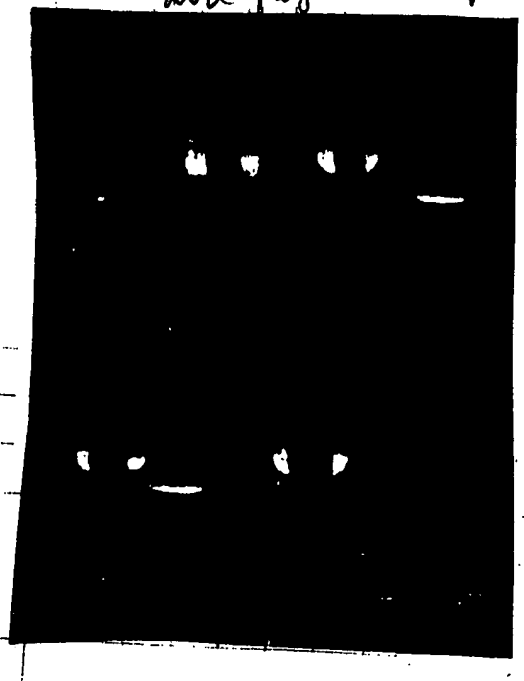


These look good as b the E and Bam site are c and gene lino for the corre size



HI
ins
p2,
PPARδ
ds
,

K3-1 to 4 dig w/ EcoR Bam and fragments cut from gel.



Ordered primers for In-fram PCR to the amp construct

Ligations to Bacterial Vectr

	K	A	
pCR II	~200	~2000	
1-ins-lig	1	0	
1-ins+lig	46	(1)	- Pick
3-ins-lig	0	0	
3-ins+lig	0	0	
1XGD	52	0	
1-XGD	44	(1)	- Pick.
3*GD	13	0	
3-XGD.	17	0	
1-T-LPL	60	0	
1-V-LPL	~140	(4)	- Pick. → Pick 10
1-T-Lep	66	(1)	
1-V-Lep	85	0	Pick 10
1-T-PPARG	49	7	
1-V-PPARG	~60	2	Pick 10
3-T-LPL	0	0	
3-V-LPL	0	0	
3-T-Lep2	1	0	
3-V-Lep2	1	(1)	- Pick.
3-T-PPARG	0	41	
3-V-PPARG	1	16	

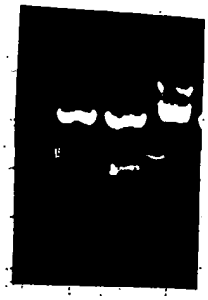
Screen w/ gene specific and one vector specific primer
Kana 5' + gene 3'

Controls Kana 5' + Kana 3'

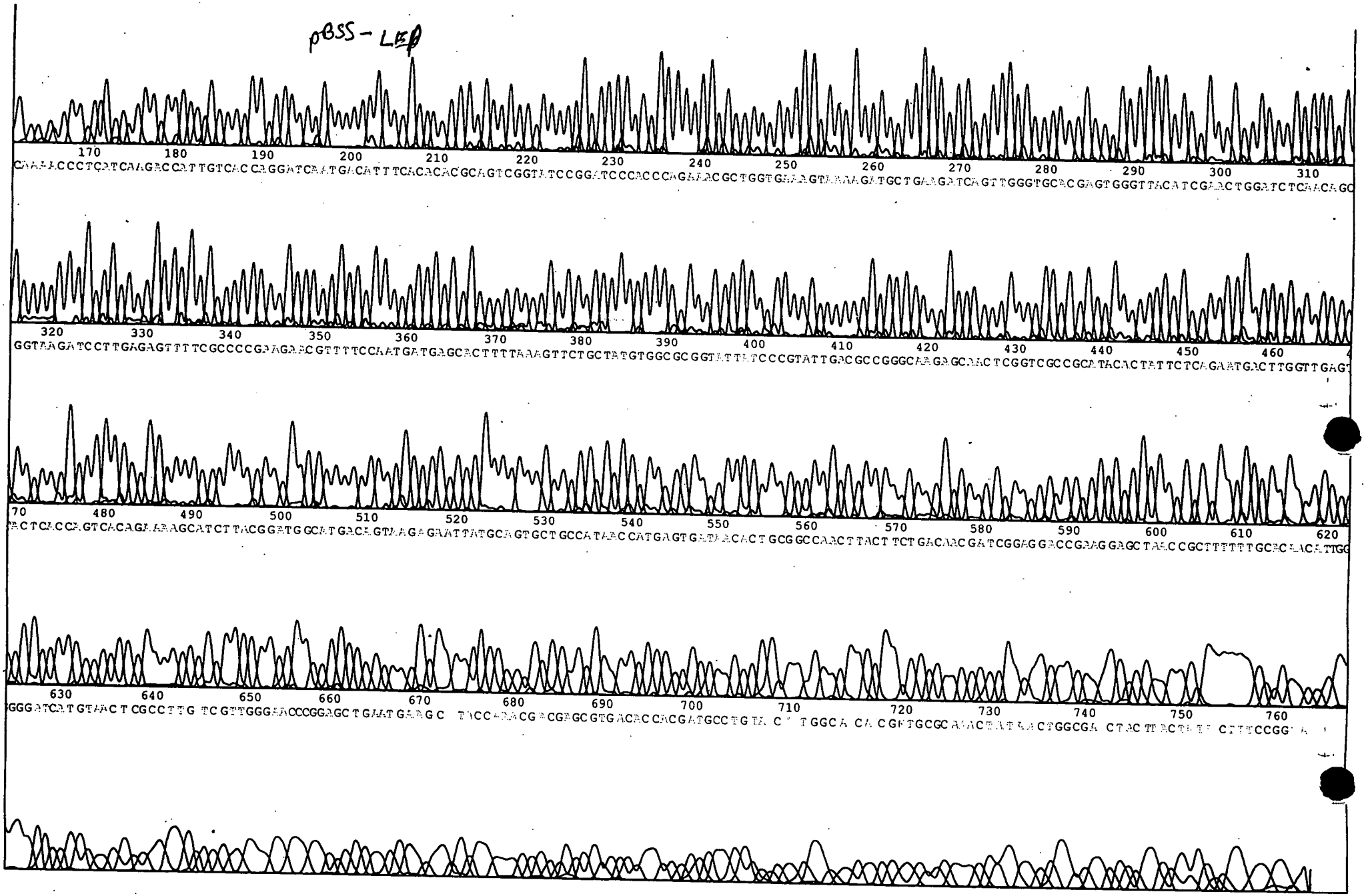
2.5 ml PCR Buffer
0.8 ml $MgCl_2$
1 ml dNTP's
1 ml primers
0.25 ml Taq.
19.5 ml water
25 ml

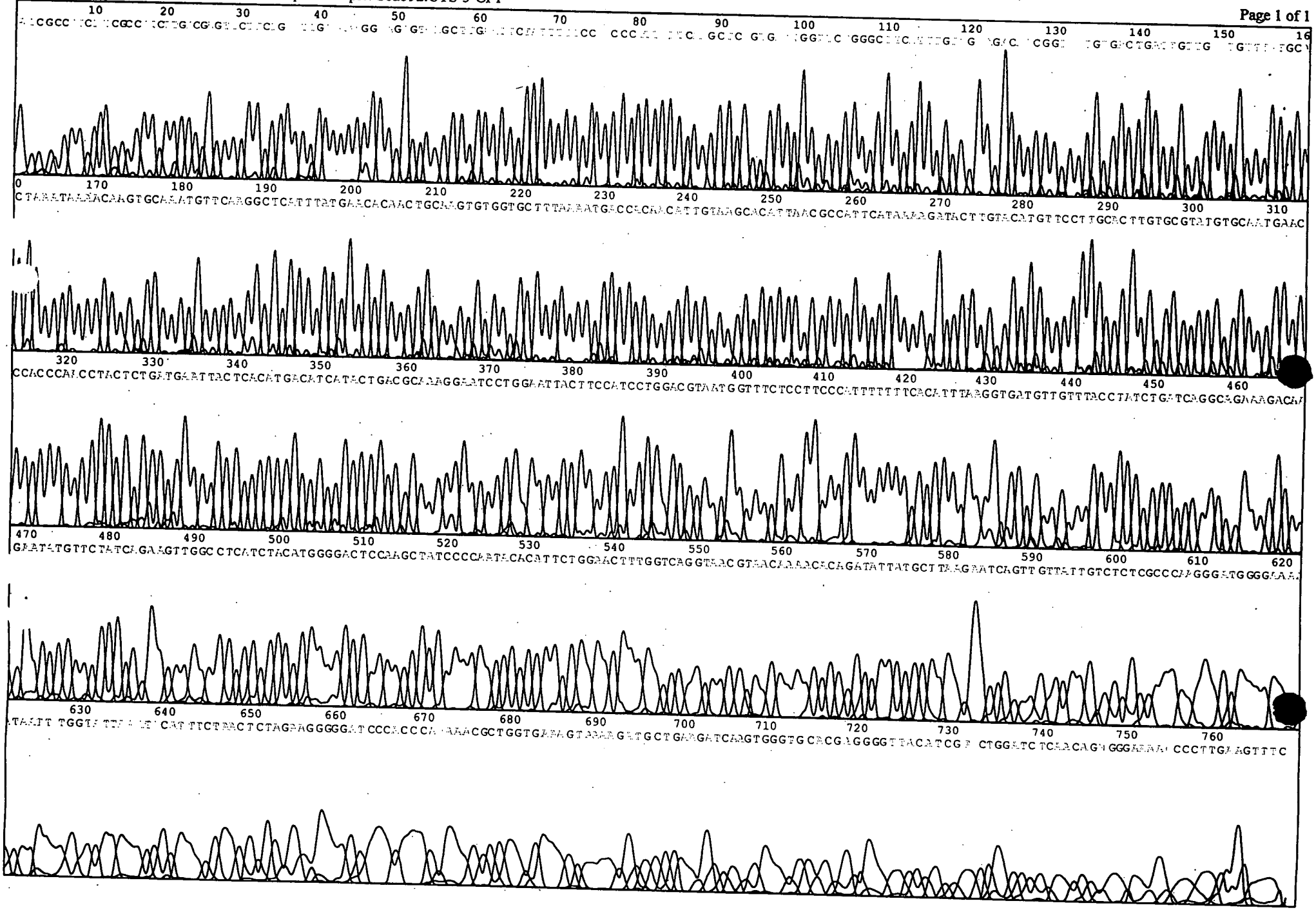
Picked colonies to LB + Kana. All the clones from the Kana plates grew but only the 1-T-Lep and the 1-XGD colonies from the amp plates grew.

Miniprepped these and digested w/ Eco/Bam



Both clones contain inserts and these were sequenced in the UTSW Core.


pBSS - LEP



e leptin fragment is exactly as I cloned it. So it appears good.

e XGD fragment contains one ORF in frame w/ the β-lactamase gene.
It codes for the following peptide.

Encoded by XGD insert.

Amp

MGKIILLLNTFLTLEWGSHPETLVKVKDAEDQLGA

euk network

Is the sequence a signal peptide?

Measure	Position	Value	Cutoff	Conclusion
max. C	21	0.325	0.37	NO
max. Y	21	0.474	0.34	YES
max. S	13	0.934	0.88	YES
mean S	1-20	0.752	0.48	YES

Most likely cleavage site between pos. 20 and 21: SHP-ET

is thus does potentially encode a secreted peptide.

then did PCR again and TA cloned the fragments of LPL, PPARδ, & Lep.
se were then cut out and geneeleaned.